Decreased expression of angiotensin-converting enzyme in the airway epithelium of asthmatic subjects is associated with eosinophil inflammation

Gabriel L. Roisman, MD, PhD,[a] Claire J. Danel, MD,[b] Jacques G. Lacronique, MD,[a] François Alhenc-Gelas, MD,[c] and Daniel J. Dusser, MD[a] *Paris, France*

Background: Angiotensin-converting enzyme (ACE) is a peptidase involved in the metabolism of several bioactive peptides. It may be involved in the airway inflammation and hyperresponsiveness that occur in asthma.
Objective: We studied the expression of ACE in the airway mucosa of normal and asthmatic subjects and assessed the relationship between ACE expression and airway inflammation and bronchial hyperresponsiveness in asthma.
Methods: We used immunohistochemistry to study the ACE expression and airway inflammation in bronchial biopsy samples obtained by fiberoptic bronchoscopy from 20 asthmatic subjects randomly assigned to groups treated with (n = 10) or without inhaled corticosteroids (n = 10) and from normal subjects (n = 10). Airway response to methacholine and bradykinin was also determined for all subjects.
Results: In normal subjects ACE was present in the surface epithelium, the endothelial cells of the lamina propria, and the submucosal glands, in which ACE was found in seromucous cells and in secreted mucus. ACE was not detected in smooth muscle cells and in most of the endothelial cells of the vascular network surrounding the glands. ACE was absent or present at lower levels in the surface epithelium of asthmatic subjects not treated with corticosteroids compared with those treated with corticosteroids and the control group. In asthmatic subjects low levels of ACE in the epithelium were associated with larger numbers of eosinophils in the epithelium and lamina propria. There was no relationship between ACE levels in the airway mucosa and airway responsiveness to methacholine and bradykinin.
Conclusion: ACE expression is decreased in the epithelium of asthmatic patients and is associated with increased eosinophil inflammation. (J Allergy Clin Immunol 1999;104:402-10.)

Key words: *Asthma, inflammation, bradykinin, kininase II, airway epithelium*

Abbreviations used

ACE:	Angiotensin-converting enzyme
APAAP:	Alkaline phosphatase antialkaline phosphatase
BAL:	Bronchoalveolar lavage
BM:	Basement membrane
LP:	Lamina propria
PD_{15}:	Dose of agonist that reduces FEV_1 by 15%
αSM actin:	α-Smooth muscle actin

Asthma is typified by bronchial hyperresponsiveness and chronic inflammation of the airway mucosa predominantly involving eosinophils.[1] Several studies have suggested that proinflammatory peptides, such as kinins and neuropeptides, may be involved in generating the chronic inflammation and airway narrowing that occur in asthma.[2,3]

Angiotensin-converting enzyme (ACE; also called kininase II, peptidyl dipeptidase A, EC 3.4.15.1) is a membrane-bound peptidase present in several types of epithelial and endothelial cells.[4] ACE is present in the lungs and airways in humans,[5] but its precise location within human airways is unknown. ACE acts as a C-terminal peptidyl dipeptidase and as an endopeptidase, converting the inactive angiotensin I into the active angiotensin II. It also inactivates a wide range of peptides, including kinins and substance P,[4,6] 2 types of proinflammatory peptides that are endogenously produced in asthma both in basal conditions and after antigen exposure.[7-9] Therefore by degrading tachykinins, kinins, and other bioactive peptides, ACE may play a key role in the mechanisms limiting the proinflammatory effects of these peptides in the airway. We have shown that ACE regulates the effects of substance P in the nasal mucosa of patients with allergic rhinitis but not in that of normal subjects.[10] A recent study has shown that patients with asthma have a higher prevalence of the DD ACE genotype, which is associated with higher plasma concentrations of ACE.[11] However, no association was found between polymorphism of the ACE gene and the severity of airway obstruction. ACE is mostly membrane bound, and therefore ACE present in airway tissue is probably physiologically more relevant than circulating ACE for the metabolism of peptides released in the airway. However, the true function of ACE in airway mucosa in humans and its potential role in asthma are unknown.

The aim of this study was to compare the distribution of ACE in the airways of normal and asthmatic subjects.

From [a]Service de Pneumologie, Hôpital Cochin, UFR Cochin Port-Royal; [b]Service d'Anatomopathologie, Hôpital Laënnec, UFR Necker-Enfants-Malades, Assistance Publique des Hôpitaux de Paris University Paris 5; and [c]INSERM U367, Paris.

Supported by a grant from INSERM (#910406).
Received for publication Jan 19, 1999; revised Apr 13, 1999; accepted for publication Apr 14, 1999.

Reprint requests: Daniel J. Dusser, MD, Service de Pneumologie, Hôpital Cochin, 27, rue du Fg Saint Jacques, 75679 Paris cedex 14, France.

0091-6749/99 $8.00 + 0 **1/1/99285**

We also investigated the relationship between ACE expression in airways and the magnitude of airway inflammation and airway hyperresponsiveness to 2 agonists, methacholine and bradykinin, the bronchoconstrictor effects of which are specific to asthma.[12]

METHODS

Subjects

Twenty nonsmokers with mild-to-moderate perennial asthma were studied. They met the American Thoracic Society's diagnostic criteria for asthma.[13] Before enrollment in the study, all patients were treated with inhaled β_2-agonist as needed, and two used theophylline regularly. With the exception of one patient who had received low doses of inhaled corticosteroids, no subjects were treated with corticosteroids for at least 1 month before inclusion into the study, and all patients were clinically stable at inclusion. Asthmatic subjects were randomly assigned to one of two groups, the first treated with inhaled β_2-agonists only if required (n = 10) and the second treated with inhaled β_2-agonists as required and inhaled flunisolide (1000 μg twice a day through a 750-mL spacer device) for 4 to 8 weeks (n = 10). Nonsmoking, nonasthmatic healthy subjects who were free of atopy were included in the study as control subjects (n = 10). None of the subjects had a respiratory infection for at least 6 weeks before or during the study or a history of occupational exposure to sensitizers. The study conformed to the Declaration of Helsinki, and it was approved by the Ethical Committee of Cochin-Port-Royal University Hospital. Written informed consent was given by all subjects.

Study design

At the preinclusion visit, each subject was interviewed and underwent chest radiography, electrocardiography, spirometry, and skin prick tests for allergies to common airborne allergens (*Dermatophagoides pteronyssinus*, *D farinae*, mixed grass pollen, mixed tree pollen, mixed weed pollen, cat fur, and dog hair). Skin prick tests were performed, and atopy was defined as previously described.[14] After inclusion, methacholine challenge, fiberoptic bronchoscopy, and bradykinin challenge were successively performed in all subjects. Bradykinin challenge was performed after bronchoscopy to prevent inflammatory changes in the airway mucosa caused by this peptide. Bronchoscopy was performed no more than 2 days after methacholine challenge and at least 5 days before bradykinin challenge. Methacholine and bradykinin challenges were performed 6 to 13 days apart. Treatment was continued on study days, and patients received no other anti-asthma drugs.

Bronchial challenges

Bronchial challenges were performed according to standard recommendations at similar times of day and according to a previously described protocol.[14] Briefly, aerosols were generated in a disposable Minineb 5610 DeVilbiss nebulizer (Somerset, Pa) by means of a breath-activated dosimeter (FDC 88, Médipron, Paris, France) under a pressure of 1.5 bar; the nebulization time was 0.6 seconds. Airway responses were assessed by measurement of FEV_1 with an automated flowmeter (Autospiro AS500, Minato, Osaka, Japan). The subjects were challenged with diluent (0.9% NaCl or PBS for methacholine and bradykinin, respectively). Cumulative doubling doses from 40 to 5080 μg (0.2 to 26.0 μmol) for methacholine and 8 to 1016 μg (0.008 to 0.958 μmol) for bradykinin were administered immediately thereafter. At least 2 satisfactory spirograms were obtained 2 minutes after each dose of methacholine. For bradykinin, spirometric responses were recorded 1, 3, and 5 minutes after each dose. The bronchial response obtained 3 minutes after each dose of bradykinin was used in all analyses because it corresponded to the maximal bronchoconstrictor effect of this agonist in our subjects and in other studies.[15] If a bronchoconstrictor response occurred, forced expiration was repeated until 2 reproducible values (FEV_1 within 5%) were obtained. Challenges were stopped when FEV_1 was at least 20% lower than the postdiluent value or when the highest dose was reached.

Bronchoscopy

Fiberoptic bronchoscopy was performed, as previously described,[14] with an Olympus BF1T20D bronchoscope (Olympus, Tokyo, Japan). Bronchoalveolar lavage (BAL) was performed after careful wedging of the bronchoscope tip into the internal segmental branch of the middle lobe bronchus. Endobronchial biopsy specimens were taken after completion of BAL from the third bifurcations and subcarinae in the left lower lobe with FB-24K or FB-37K forceps (Olympus). Biopsy specimens were gently extracted from the forceps and immediately fixed in Bouin's fluid and/or frozen in liquid nitrogen and stored at –80°C.

Analysis of inflammatory cells in BAL fluid

BAL fluid was shaken gently to resuspend the cells, and the volume was measured. Cells were sedimented by cytocentrifugation onto slides and stained with a modified Wright-Giemsa stain; differentials for cell counts were made by light microscopy. For each individual sample, 1000 cells chosen at random were analyzed.

Morphometric analysis of bronchial biopsy specimens

For each subject, 2 or 3 bronchial biopsy specimens were fixed in Bouin's fluid, embedded in paraffin, and cut into 4-μm thick sections. The sections were stained with hematoxylin eosin or Luna's reagent, which is specific for eosinophils.[14,16] Observer bias was prevented by coding of the slides, which were examined without prior knowledge of the state of the individual subject. Bronchial mucosa were assessed by integral analysis with a light microscope (Leitz Aristoplan; Rueil-Malmaison, France). A computerized imaging system (Morphostar 4.01 video analysis software; Instar, Paris, France) was used to evaluate various histologic features: (1) the extent of inflammatory cell infiltration, (2) subepithelial fibrosis, and (3) the shedding of the epithelium. The lamina propria (LP) was arbitrarily taken to be a zone 50 μm beneath the reticular basement membrane (BM). Intact epithelium was defined as epithelium with both basal and columnar cells. Shedding of the airway epithelium was expressed as the percentage of the length of BM in the section covered by intact epithelium (millimeters of intact epithelium/millimeters of BM × 100).

Assessment of inflammatory cell infiltrates. Infiltration by lymphocytes and eosinophils was expressed as the number of cells per square millimeter of LP and the epithelium. Neutrophils were not counted because they were rare or absent in our specimens.

Assessment of the thickness of subepithelial fibrosis. The thickness of subepithelial fibrosis was assessed, as previously described.[17] The distance from the base of the bronchial epithelium to the outer limit of the reticular lamina of the BM was determined at 200-μm intervals along the length of each section. The result was expressed as the mean of 10 measurements per section.

Immunohistochemical detection of ACE in bronchial biopsy specimens

Freshly obtained bronchial biopsy specimens were embedded in Tissue-Tek OCT compound (Miles Inc), frozen in isopentane precooled in liquid nitrogen, and stored at –80° C. Cryostat sections (6-μm thick) were cut from the cryopreserved tissue blocks and fixed

TABLE I. Characteristics of subjects

	Asthmatic subjects treated without corticosteroids	Asthmatic subjects treated with corticosteroids	Normal subjects
Sex (M/F)	7/3	8/2	5/5
Age (y)	33.8 ± 4.5	30.8 ± 3.3	26.3 ± 2.0
Duration of asthma (y)	14.6 ± 2.3	12.2 ± 2.5	—
Atopy (n)	9	9	0
FEV_1 (% predicted)	92.2 ± 4.5	89.2 ± 4.1	101.3 ± 3.6
$PD_{15}FEV_1$ methacholine (μmol)	0.78 (0.12-13.78)	0.98 (0.05-16.68)	NR
$PD_{15}FEV_1$ bradykinin (μmol)	0.073 (0.003-0.873)	0.144 (0.008-0.815)	NR

Results are given as geometric medians (range) or as means ± SEM.
NR, Not reactive.

in acetone for 10 minutes. Two primary antibodies recognizing human ACE were used: the mouse mAb 9B9 (kindly provided by Dr S. M. Danilov)[18] and the rabbit polyclonal antibody CLO (kindly provided by Dr F. Alhenc-Gelas).[19] Nonspecific binding sites were blocked with a 1:20 (vol/vol) dilution of normal rabbit serum (X902; DAKO, Glostrup, Denmark) or normal mouse serum (X910; DAKO). Sections were then incubated with primary antibodies raised in mice or rabbits, respectively. Incubation with the rabbit polyclonal primary antibody CLO was followed by a 30-minute incubation with mouse anti-rabbit monoclonal mouse antibody (M737; DAKO). For each subject, serial sections were incubated overnight at 4°C with appropriate dilutions of primary antibody. Parallel sections were used as negative controls, and for these sections, the primary antibody was replaced by the same concentration of an isotype-matched antibody (DAK-GO1, X931; DAKO). Antibodies were diluted in Tris-buffered saline (pH 7.6) containing 1% BSA. Sections were washed in Tris-buffered saline, and antibody binding was detected by alkaline phosphatase antialkaline phosphatase (APAAP) technique (Universal APAAP Kit for mouse primary antibody, K670; DAKO) or the avidin-biotin complex (Vectastain Elite ABC kit; Vector, Burlingame, Calif) methods. Fast red or diaminobenzidine with hydrogen peroxide, respectively, were used as enzyme substrates. Sections were counterstained with hematoxylin and mounted. Cryostat sections of human lung parenchyma were used as positive tissue controls for the primary antibodies. Coded sections of the bronchial mucosa were assessed by 2 investigators with no prior knowledge of subject status.

Assessment of the distribution of ACE. To determine the phenotype of cells expressing ACE, we compared the distribution of this peptidase to that of other markers. Thus other serial sections were stained with the mouse mAb JC/70A against the human endothelial cell marker, the CD31 antigen (M 823; DAKO); the mouse mAb MNF116 against human cytokeratin (M 821; DAKO); the mouse mAb 1A4 against human α-smooth muscle actin (αSM actin; M 851; DAKO); and the mouse mAb against human vimentin (M 725; DAKO), a marker of cells of mesenchymal origin. Endothelial, epithelial, and smooth muscle cells were identified by signals with antibodies against CD31, cytokeratin, and αSM actin, respectively. Spindle-shaped cells in the LP, which gave a signal for vimentin but not for CD31 or αSM actin, were classified as fibroblasts. Myoepithelial cells were identified by immunohistochemistry, morphology, and location. Myoepithelial cells in the seromucous glands were identified as spindle-shaped cells containing both cytokeratin and αSM actin located between the basal edge of secretory epithelial cells and the BM.

Assessment of the degree of ACE immunoreactivity. The degree (intensity and pattern) of ACE immunoreactivity in sections stained with the 9B9 mAb was assessed by 2 researchers using a semiquantitative scoring system (0 = none, 1 = weak, 2 = moderate, and 3 = strong), and a mean score was calculated for each subject. ACE immunolabeling of the epithelium was scored and defined as low (scores 0 and 0.5), intermediate (scores 1 and 1.5), or high (score ≥2). To avoid any possible mistake because of damaged or reduced epithelium in asthmatic subjects, we have assessed the score of ACE labeling only in intact epithelium in all subjects. For technical reasons, scoring of ACE immunoreactivity in the surface of epithelium was performed in 9 of 10 control subjects and 17 of 20 patients with asthma (8 with and 9 without inhaled corticosteroids).

Data analysis

FEV_1 is expressed as a percentage of predicted values. Bronchial responses to agonists are expressed as the cumulative dose causing a 15% decrease in FEV_1 from the postdiluent value (PD_{15}). This was derived by linear interpolation from the dose-response curve, according to previously defined guidelines.[20] If the maximal decrease in FEV_1 was less than 15% after the last dose of the agonist was given, the subject was considered to be not reactive to that agonist. PD_{15} was used in our study because the maximum decrease in FEV_1 was less than 20% for bradykinin challenge in 6 asthmatic subjects.

Group data are expressed as the median (range) for differential cell counts in the BAL fluid and for biopsy findings because these variables could not be demonstrated to have a normal distribution. For subjects responsive to agonist challenges, methacholine and bradykinin PD_{15} values are expressed as the geometric mean (range) because these variables are known to have a log-normal distribution. All other variables are expressed as means ± 1 SEM. Nonparametric tests were used for variables involving BAL or biopsy data. For these variables, comparisons between groups were made by using the Kruskal-Wallis test, and *P* values were corrected for ties. For the other variables, groups were compared by ANOVA or unpaired Student *t* tests as appropriate. Nonparametric and parametric post hoc comparisons (pairwise differences) were made as required by using a Tukey-type multiple comparison test or Scheffé's multiple contrast test, respectively.[21] Probability *(P)* values of .05 or less were considered to be statistically significant.

RESULTS

Clinical characteristics of subjects

The clinical characteristics of the 30 subjects studied are shown in Table I. Groups did not differ significantly regarding the age of the subjects or FEV_1 at inclusion. Most subjects with asthma were atopic (9 subjects in each group). Normal subjects did not react to methacholine or bradykinin, whereas most of the asthmatic patients reacted to both agonists. Methacholine and bradykinin PD_{15} values did not differ between the 2 groups of asthmatic patients.

TABLE II. BAL cytology

	Normal subjects	Asthmatic subjects treated with corticosteroids	Asthmatic subjects treated without corticosteroids
Eosinophils (%)	0.1 (0.0-1.0)	0.4 (0.0-21.2)	1.0 (0.0-6.4)*
Neutrophils (%)	1.8 (0.0-2.2)	1.6 (0.1-3.9)	1.4 (1.0-13.6)
Lymphocytes (%)	8.9 (4.0-20.6)	10.5 (2.0-31.0)	9.2 (3.9-28.9)
Macrophages (%)	89.3 (77.1-96.0)	83.4 (64.0-96.0)	85.5 (67.7-95.0)

Results are given as median (range).
*$P < .05$ for comparison with normal subjects.

TABLE III. Bronchial biopsy

	Normal subjects	Asthmatic subjects treated with corticosteroids	Asthmatic subjects treated without corticosteroids
Thickness of subepithelial fibrosis (μm)	4.7 (3.2-9.5)	6.3 (4.3-10.3)*	7.6 (6.9-12.7)*
BM covered by epithelium (%)	45.7 (6.4-92.0)	34.8 (5.3-66.7)	18.0 (0.0-57.4)*
Eosinophils in epithelium and LP (cells/mm^2)	0.0 (0.0-43.3)	1.5 (0.0-12.4)	28.5 (0.0-1337.9)*†
Lymphocytes in epithelium and LP (cells/mm^2)	556 (194-1306)	389 (194-2944)	1583 (278-3111)*†

Results are given as median (range).
*$P < .05$ for comparison with normal subjects.
†$P < .05$ for comparison with asthmatic subjects treated with corticosteroids.

Evaluation of inflammation in BAL fluid and in bronchial biopsy specimens

The data for differential inflammatory cell counts in BAL fluid are shown in Table II. The percentage of eosinophils in BAL fluid was higher in asthmatic patients not treated with corticosteroids than in normal subjects ($P < .03$), whereas the percentages of eosinophils in normal subjects and asthmatic patients treated with corticosteroids were similar.

The results of the morphometric analysis are shown in Table III. There were no differences between asthmatic subjects taking and not taking corticosteroids and normal subjects in the total length of epithelium (8.51 ± 1.06, 6.49 ± 1.17, and 7.90 ± 1.26 mm of epithelium, respectively) and total area of bronchial mucosa (0.18 ± 0.04, 0.16 ± 0.03, and 0.21 ± 0.05 mm^2, respectively) examined. Asthmatic subjects not treated with corticosteroids had more eosinophils and lymphocytes in the surface epithelium and LP than did asthmatic subjects treated with corticosteroids and normal subjects ($P < .05$ in both cases). Shedding of the airway epithelium was more extensive in asthmatic subjects not treated with corticosteroids than in normal subjects ($P < .05$) but occurred to a similar extent in the 2 groups of asthmatic subjects. There was a thicker layer of subepithelial fibrosis in both groups of asthmatic subjects than in normal subjects ($P < .05$ in each case), but the extent of subepithelial fibrosis was similar for the 2 groups of asthmatic subjects.

Immunohistochemical analysis of ACE in bronchial biopsy specimens

Distribution of ACE in normal subjects. The distribution of ACE as detected by immunostaining was similar in sections stained with 9B9 and CLO antibodies. In the bronchial wall ACE was present in both the surface epithelial cells (Fig 1, *A*) and the epithelial cells of the submucosal glands (Fig 1, *B*). In the submucosal glands ACE was present in serous and mucous epithelial cells (Fig 1, *B*) and was also detected in the secreted material within the acini (Fig 1, *C*). ACE was also detected in endothelial cells. In particular, a large amount of ACE was expressed by CD31-positive endothelial cells of the subepithelial capillovenular plexus beneath the BM (Fig 1, *A*). In the submucosa ACE was detected in all the endothelial cells of the vessels surrounding the αSM actin–positive smooth muscle cells (not shown). In contrast, ACE was rarely detected in the endothelial cells of the vascular network surrounding the acini of the submucosal glands, regardless of the caliber of the vessel. ACE was not detected in the BM (Fig 1, *A*), smooth muscle cells, or fibroblasts. The intensity of ACE staining was generally higher in endothelial cells than in epithelial cells.

Control subjects with an irrelevant isotype-matched mAb were negative in each case.

ACE distribution in asthmatic subjects. The amount of ACE in surface epithelial cells was lower in asthmatic subjects not treated with corticosteroids (Fig 2) than in normal subjects (Fig 1, *A*). ACE-labeling scores for the epithelium were similar for the 2 investigators (Wilcoxon paired comparison, $P > .99$). Thus in sections containing airway epithelium, the intensity of immunostaining for ACE was lower in asthmatic patients not treated with inhaled corticosteroids than in patients treated with inhaled corticosteroids and normal subjects (each comparison, $P < .01$; Fig 3). ACE staining in parts of the airway mucosa other than the epithelium was similar for asthmatic and normal subjects.

Relationship between ACE expression in the epithelium and airway inflammation and bronchial responsiveness in asthmatic subjects

The number of eosinophils in the epithelium and LP differed significantly among patients with low, intermediate, or high scores for ACE in the epithelium ($P < .05$;

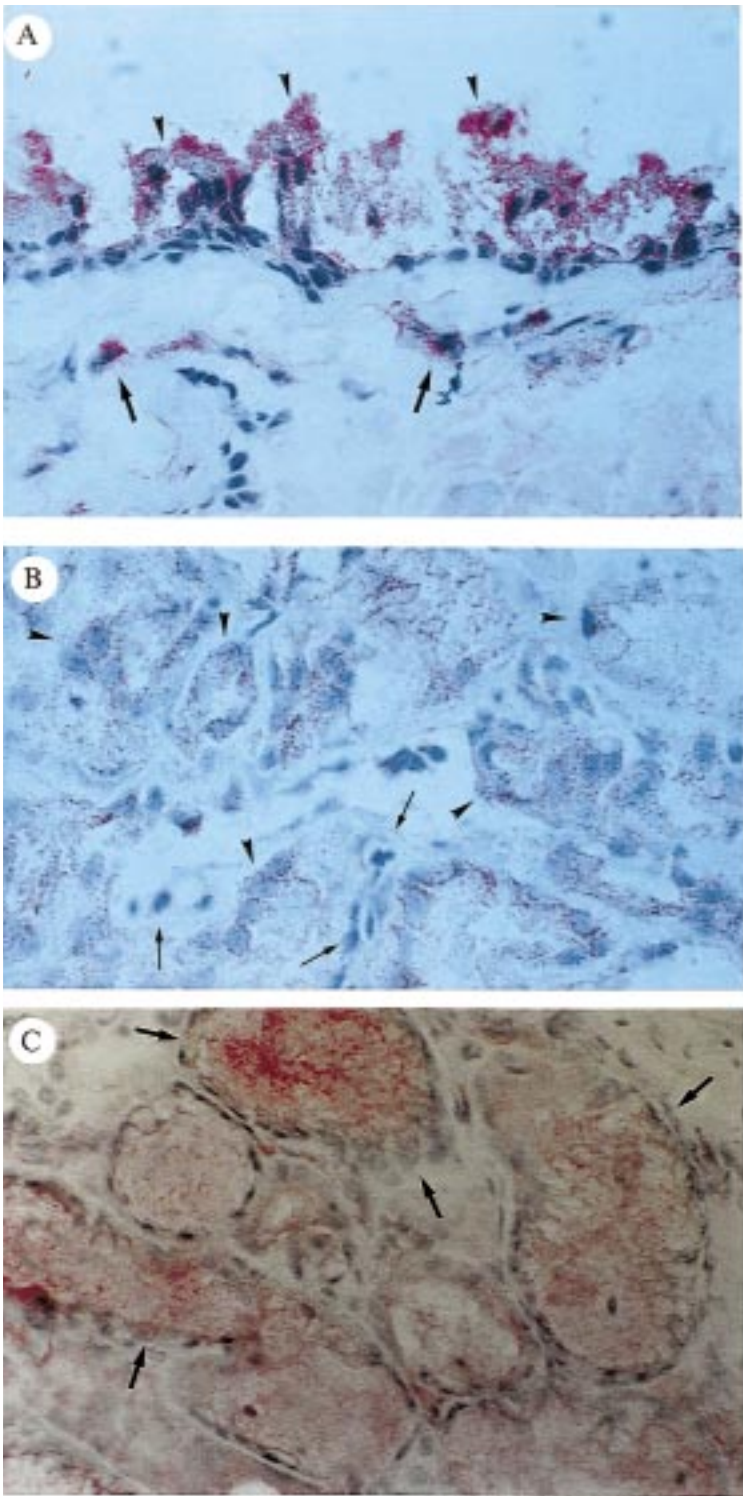

FIG 1. Photomicrographs showing the distribution of ACE in human endobronchial biopsy samples obtained by fiberoptic bronchoscopy in control subjects. ACE is stained by a pink-red color. **A,** High magnification of bronchial mucosa stained with ACE mAb (9B9). Surface epithelium is labeled *(arrowheads)*, as are the endothelial cells of the LP capillaries *(arrows)*. **B,** Immunohistochemical detection of ACE in the submucosal gland area. The epithelial glandular cells are labeled *(arrowheads)*, whereas the connective tissue surrounding the glands is not. This is particularly true for the endothelial cells *(arrows)*. **C,** Same as **B,** with ACE (red signal) present in both glandular epithelial cells *(arrowheads)* and intraluminal secretions. All samples were evaluated by immunohistochemistry by using the APAAP technique and hematoxylin counterstaining (all panels, original magnification ×400).

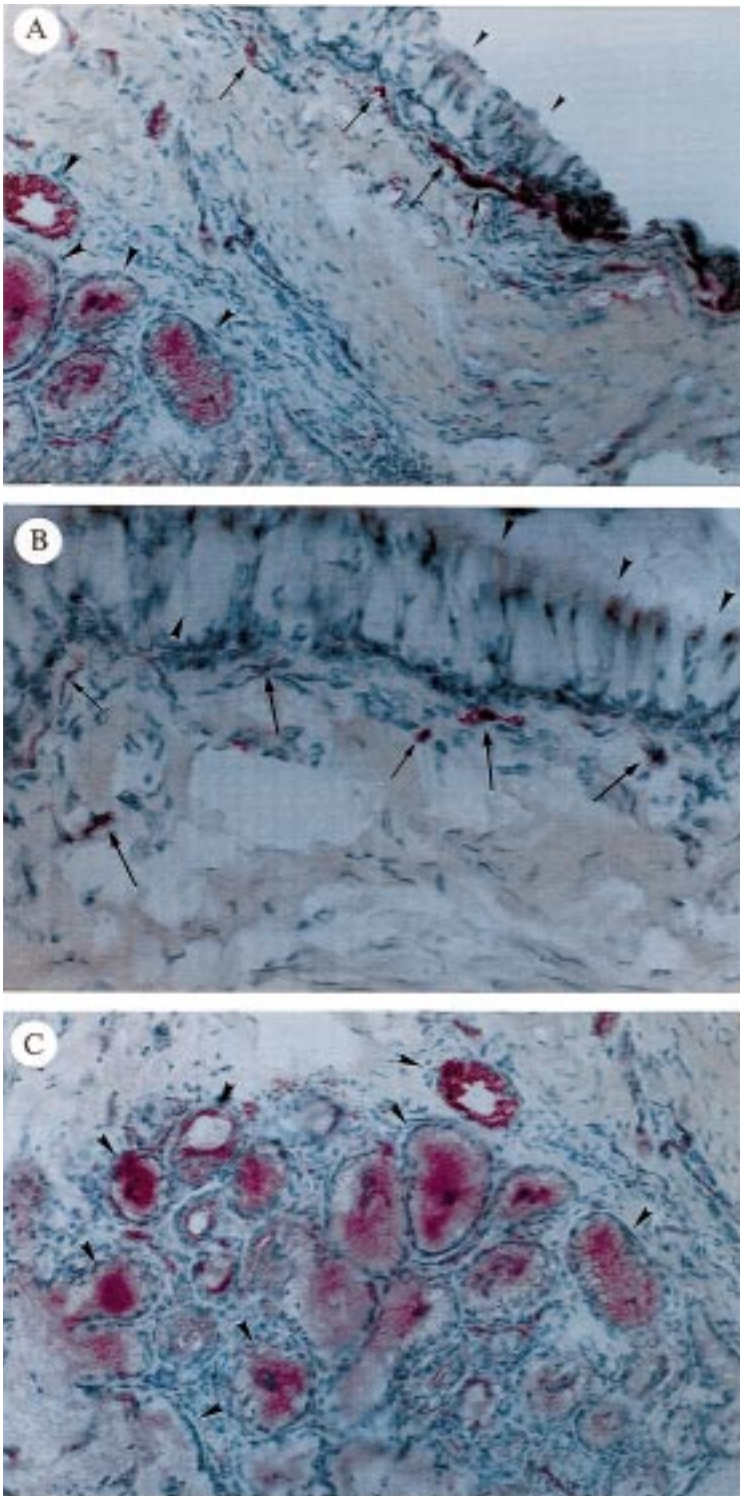

FIG 2. ACE detected by immunochemistry in an endobronchial biopsy sample from an asthmatic patient not treated with corticosteroids. **A,** In contrast to control subjects, there is little expression of ACE in the surface epithelium *(thin arrowheads)* and larger amounts in the endothelial cells beneath the BM *(arrows)* and submucosal glands *(arrowheads)*. **B,** Same as **A,** with higher magnification of the ACE-negative surface epithelium *(arrowheads)* contrasting with the ACE-positive LP endothelial cells *(arrows)*. **C,** Same as **A**, with higher magnification of the submucosal gland area. ACE (red signal) was detected in both epithelial gland cells and intraluminal secretions *(arrowheads)*. All samples were evaluated by immunohistochemistry by using the APAAP technique and hematoxylin counterstaining (**A,** original magnification ×180; **B** and **C,** original magnification ×400).

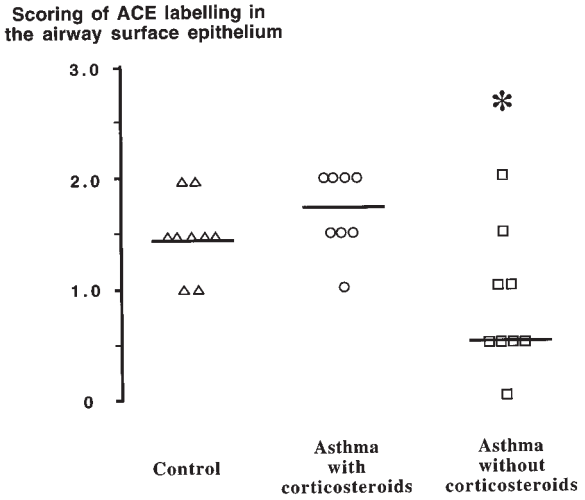

FIG 3. Intensity of immunostaining for ACE with ACE mAb (9B9) in the surface epithelium of normal subjects and asthmatic subjects treated or not treated with inhaled corticosteroids. ACE labeling was scored 0 to 3 by histologic assessment on a semiquantitative scale. *$P < .05$ for comparisons between asthmatic patients not treated with inhaled corticosteroids and the 2 other groups.

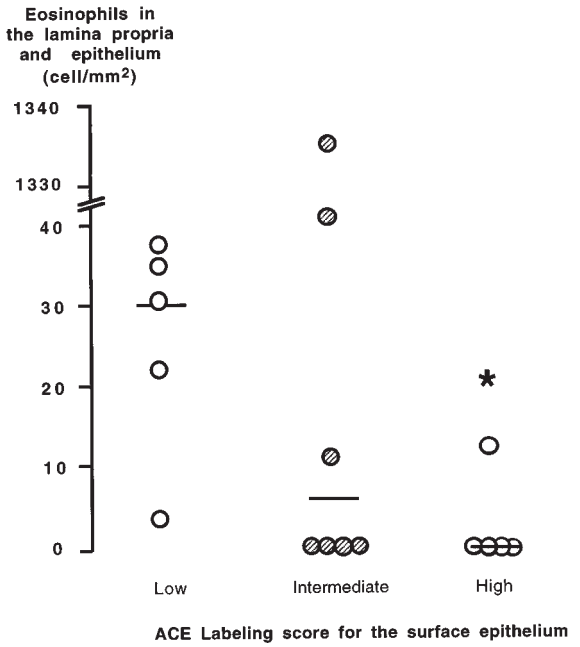

FIG 4. Relationship between the number of eosinophils in the epithelium and LP and ACE-labeling score in the surface epithelium of asthmatic subjects. ACE-immunolabeling score was classified as low (score 0 or 0.5), intermediate (score 1 or 1.5), or high (score 2). *Bars* are the median numbers of eosinophils in each group of ACE scores. The number of eosinophils increased with decreasing ACE score in the surface epithelium ($P < .05$). Eosinophil infiltration was lowest in patients with high ACE scores in the surface epithelium (*$P < .05$).

Fig 4). The number of eosinophils in the bronchial mucosa decreased with increasing levels of ACE score in the surface epithelium; eosinophil inflammation was greatest in patients with low scores for ACE in the epithelium. There was no relationship between the intensity of ACE staining in the epithelium and the number of lymphocytes in the airway mucosa, the extent of subepithelial fibrosis, or BAL results.

No relationship was found between the ACE score for the epithelium and the PD_{15} to methacholine and bradykinin ($P > .05$ for each).

DISCUSSION

We demonstrated the presence of ACE in large airways of both normal and asthmatic subjects, with low expression of ACE in the surface epithelium of asthmatic patients not treated with corticosteroids. In a previous study investigating the distribution of ACE in the human lung, Johnson et al[5] found that this peptidase was present mostly in endothelial cells. This study is the first to investigate the distribution of ACE in large airways in human subjects and to provide evidence that it is present in airway epithelial cells. In the mucosa ACE was detected in both the surface epithelium and the endothelial cells of the subepithelial capillovenular plexus beneath the BM. In the submucosa ACE was present mainly in the submucosal glands, in the seromucous cells, and in the mucus secreted within the acini. ACE was also detected in the endothelial cells of the bronchial vessels around the smooth muscle but not in the vessels surrounding the submucosal glands.

The physiologic function of ACE in normal human airways is unknown. However, ACE is known to degrade several bioactive peptides in other cells or organs. Its presence in the airways may have several important physiologic implications. In epithelial cells ACE may regulate the effects of endogenously produced kinins and tachykinins released from intraepithelial C-fiber nerves.[22] These nerves are present in high density in the environment of the surface epithelium.[23] Therefore ACE is probably involved in the regulation the proinflammatory effects of the bioactive peptides that are released in response to various stimuli of the surface epithelium or intraepithelial nerves, such as irritants. ACE in the surface epithelium may also regulate the cough reflex caused by some peptides, including kinins.[24] The inhibition of ACE in the airway epithelium may account for the high frequency of coughing as an adverse effect of ACE inhibitor treatment in humans.[25] In airway endothelial cells ACE may be involved in the regulation of peptide-induced vasodilation and microvascular leakage. ACE was also present in submucosal glands. These glands are highly innervated by neuropeptide-containing nerves[26] and are the major source of tissue kallikrein, which is responsible for the formation of kinins from plasma precursors.[7,8] Thus ACE in epithelial gland cells may regulate the mucus secretion induced by neuropeptides and kinins at the site of release of these molecules.[2,3] ACE was present in the secreted mucus, suggesting that there

is a soluble extracellular form of the peptidase, probably originating from the seromucous cells of the submucosal glands. Such a soluble, extracellular form of the peptidase has been described in other types of human fluid.[27,28] ACE may degrade peptide mediators or peptide constituents of the mucus in the glands or the airway lumen. Finally, ACE was not present in airway smooth muscle, suggesting that this peptidase is not involved in the regulation of smooth muscle contraction in large airways.

ACE expression was reduced in the surface epithelium of asthmatic patients not treated with corticosteroids. Reduced or damaged epithelium in asthmatic patients may affect the analysis of morphometric data. To avoid this problem we have analyzed the intensity of ACE labeling only in intact epithelium in all subjects. In addition, we have examined a similar length of surface epithelium and a similar surface area of mucosa in each group of subjects. Therefore scoring of ACE labeling reflects the expression of the peptidase in the intact epithelium and is not dependent on the amount of shedding of the epithelium. Thus reduced or damaged epithelium cannot account for the reduced ACE expression found in asthmatic subjects not treated with corticosteroids. Using techniques that we have described earlier,[29] we measured activity in homogenates of bronchial biopsy specimens from our subjects. We found no difference in ACE activity between the groups of patients (data not shown). However, ACE is expressed in several different cell types in the airway mucosa, some of which contain a high intensity of activity (eg, vessels). Therefore measuring activity of ACE in the whole homogenate of airway mucosa reflects the presence of ACE in all cells. This makes it unlikely that differences in ACE activity will be detected in cells such as those in the surface epithelium, where the relative contribution of the total ACE activity is much lower than that in other tissues, such as vessels. Low levels of ACE in the epithelium were associated with increased eosinophil inflammation of the airway mucosa. It is possible that eosinophils that have toxic oxidizing effects may be involved in the degradation of the peptidase in the epithelium. Alternatively, low levels of ACE in the epithelium may limit degradation of a bioactive peptide with a chemotactic effect on eosinophils. Substance P is a possible candidate for this function because it is naturally produced in asthma and is known to stimulate the migration and activation of eosinophils.[30-32] Low levels of ACE in the surface epithelium may have several other consequences, which are difficult to evaluate fully because this peptidase acts on a large variety of peptides, some of which are unknown.

The reasons for the low expression of ACE in the surface epithelium of asthmatic subjects not treated with corticosteroids are unclear. ACE may be destroyed by oxidants,[33] or epithelial cells may synthesize less of this protease in such patients. ACE levels in the airway epithelium were similar in normal and asthmatic subjects treated with corticosteroids, suggesting that this treatment restores and maintains the levels of this protease in the airway surface epithelial cells. This may be due to the anti-inflammatory effects of corticosteroids, reducing both eosinophil number and activation and thereby reducing the deleterious effects of eosinophils on ACE. Alternatively, corticosteroids may directly stimulate the synthesis of ACE by epithelial cells, as was shown for other cells.[34] The influence of corticosteroids on the expression of ACE by the surface epithelium in asthmatic subjects is to be put together with the demonstration, in animals, that ACE regulates the inhibitory effects of corticosteroids on neurogenic inflammation.[35,36] These findings suggest that the interaction between ACE and corticosteroids may be one of the mechanisms through which this treatment is beneficial to patients with asthma.

Asthmatic patients have an increased responsiveness to bradykinin.[3] Although bradykinin is one of the principal substrates of ACE,[4,6] our results do not support the hypothesis that reduced levels of ACE in the surface epithelium are responsible for the increased bronchoconstrictor response to bradykinin in asthma. The direct action of bradykinin on the airway smooth muscle is weak. Instead, bradykinin causes bronchoconstriction by means of complex, indirect actions on vessels[37,38] and mucus secretion[39] by means of cholinergic and peptidergic reflexes.[24] This complexity of the mechanisms of responses to bradykinin makes it very difficult to draw definitive conclusions about the lack of relationship between ACE levels and response to bradykinin for our population of asthmatic subjects. Because we needed to perform fiberoptic bronchoscopies and bronchial challenges, it was necessary to study patients with mild, stable asthma. Thus even though we found a greater number of eosinophils and lymphocytes in patients not treated with corticosteroids, pulmonary function test results were normal in asthmatic patients treated with and without corticosteroids, and no clinical differences existed between these 2 groups of patients. The selection of this group of patients with mild, stable asthma is likely to explain the lack of correlation between ACE expression and clinical data. The relationship between ACE expression and the severity of asthma needs to be addressed by designing another study that includes patients with various asthma severities.

Thus ACE is widely distributed in the airway mucosa of large airways of normal subjects, particularly in the surface epithelium, submucosal gland cells, and secretory material. The distribution of ACE is consistent with its regulating the effects of tachykinins, kinins, and other peptide mediators locally produced in the airway mucosa. ACE expression is reduced in the surface epithelium of asthmatic subjects not treated with inhaled corticosteroids, whereas treatment with corticosteroids restores ACE levels in the epithelium to those of normal subjects. Low levels of expression of ACE in the airway epithelium are associated with an increased eosinophil infiltration of the airway mucosa. ACE is active against a large variety of peptides, some of which are unknown. Therefore low levels of ACE in the airway epithelium may have other

major effects on the pathologic physiology of asthma in addition to being associated with increased eosinophil inflammation.

We thank Dr S. M. Danilov for providing us with the ACE antibody and for his comments and expertise in kinase II research.

REFERENCES

1. Djukanovic R, Roche WR, Wilson JW, Beasley CRW, Twentyman OP, Howarth PH, et al. Mucosal inflammation in asthma. Am Rev Respir Dis 1990;142:434-57.
2. Barnes PJ, Baraniuk JN, Belvisi MG. Neuropeptides in the respiratory tract. Part II. Am Rev Respir Dis 1991;144:1391-9.
3. Barnes PJ. Bradykinin and asthma. Thorax 1992;47:979-83.
4. Erdös E. Angiotensin I converting enzyme and the changes in our concepts through the years. Hypertension 1990;16:363-70.
5. Johnson AR, Ashton J, Schulz WW, Erdös EG. Neutral metalloendopeptidase in human lung tissue and cultured cells. Am Rev Respir Dis 1985;132:564-8.
6. Skidgel RA, Erdös EG. The broad substrate specificity of human angiotensin I converting enzyme. Clin Exp Hypertens 1987;A9:243-59.
7. Christiansen SC, Proud D, Cochrane CG. Detection of tissue kallikrein in bronchoalveolar lavage fluid of asthmatic subjects. J Clin Invest 1987;79:188-97.
8. Christiansen SC, Proud D, Sarnoff RB, Juergens U, Cochrane CG, Zuraw BL. Elevation of tissue kallikrein and kinin in the airways of asthmatic subjects after endobronchial allergen challenge. Am Rev Respir Dis 1992;145:900-5.
9. Nieber K, Baumgarten CR, Rathsack R, Furkert J, Oehme P, Kunkel G. Substance P and β-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. J Allergy Clin Immunol 1992;90:646-52.
10. Lurie A, Nadel J, Roisman G, Siney H, Dusser D. Role of neutral endopeptidase and angiotensin converting enzyme in the modulation of the nasal response to substance P in patients with allergic rhinitis and in healthy volunteers. Am J Respir Crit Care Med 1994;149:113-7.
11. Benessiano J, Crestani B, Mestari F, Klouche W, Neukirch F, Hacein-Bey S, et al. High frequency of deletion polymorphism of the angiotensin-converting enzyme gene in asthma. J Allergy Clin Immunol 1997;99:53-7.
12. Cockcroft DW, Hargreave FE. Airway hyperresponsiveness: definition, measurement, and clinical relevance. In: Kaliner MA, Barnes PJ, Persson CGA, editors. Asthma: its pathology and treatment. New York: Marcel Dekker, Inc; 1991. p. 51-72.
13. American Thoracic Society. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am Rev Respir Dis 1987;136:225-43.
14. Roisman GL, Lacronique JG, Desmazes-Dufeu N, Carré C, Le Cae A, Dusser DJ. Airway responsiveness to bradykinin is related to eosinophilic inflammation in asthma. Am J Respir Crit Care Med 1996;153:381-90.
15. Polosa R, Holgate ST. Comparative airway responses to inhaled bradykinin, kallidin, and [des-Arg9] bradykinin in normal and asthmatic subjects. Am Rev Respir Dis 1990;142:1367-71.
16. McElroy DA. Laboratory methods in histotechnology. 1st ed. Washington (DC): American Registry of Pathology; 1992.
17. Saetta M, Maestrelli P, Turato G, Mapp CE, Milani G, Pivirotto F, et al. Airway wall remodeling after cessation of exposure to isocyanates in sensitized asthmatic subjects. Am J Respir Crit Care Med 1995;151:489-94.
18. Danilov SM, Faerman AI, Printseva OY, Martynov AV, Sakharov IY, Trakht IN. Immunohistochemical study of angiotensin-converting enzyme in human tissues using monoclonal antibodies. Histochemistry 1987;87:487-90.
19. Sibony M, Gasc J-M, Soubrier F, Alhenc-Gelas F, Corvol P. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. Hypertension 1993;21:827-35.
20. Eiser NM, Kerrebijn KF, Quanjer PH. SEPCR working group "bronchial hyperreactivity": guidelines for standardization of bronchial challenges with (nonspecific) bronchoconstricting agents. Bull Eur Physiopathol Respir 1983;19:495-514.
21. Zar JH. Biostatistical Analysis. Englewood Cliffs (NJ): Prentice-Hall; 1984.
22. Ichinose M, Nakajima N, Takahashi T, Yamauchi H, Inoue H, Takishima T. Protection against bradykinin-induced bronchoconstriction in asthmatic patients by neurokinin receptor antagonist. Lancet 1992;340:1248-51.
23. Lundberg J, Hökfelt T, Martling C-R, Saria A, Cuello C. Substance P-immunoreactive sensory nerves in the lower repiratory tract of various mammals including man. Cell Tissue Res 1984;235:251-61.
24. Fuller R, Dixon C, Cuss F, Barnes P. Bradykinin-induced bronchoconstriction in humans. Mode of action. Am Rev Respir Dis 1987;135:176-80.
25. Coulter D, Edwards I. Cough associated with captopril and enalapril. BMJ 1987;294:1521-3.
26. Lucchini R, Facchini F, Turato G, Saetta M, Caramori G, Ciaccia A, et al. Increased VIP-containing fibers in the mucous glands of subjects with chronic bronchitis. Am J Respir Crit Care Med 1997;156:1963-8.
27. Costerousse O, Jaspard E, Alhenc-Gelas F. Molecular and genetic aspects of dipeptidyl carboxypeptidase I (the angiotensin I converting enzyme). Expression in the immune system. Adv Neuroimmunol 1993;3:217-26.
28. Spillantini MG, Sicuteri F, Salmon S, Malfroy B. Characterization of endopeptidase 3.4.24.11 ("enkephalinase") activity in human plasma and cerebrospinal fluid. Biochem Pharmacol 1990;39:1353-6.
29. Dusser DJ, Nadel JA, Sekizawa K, Graf PD, Borson DB. Neutral endopeptidase and angiotensin converting enzyme inhibitors potentiate kinin-induced contraction of ferret trachea. J Pharmacol Exp Ther 1988;244:531-6.
30. Kroegel C, Giembycz M, Barnes P. Characterization of eosinophil activation by peptides. Differential effects of substance P, melitin, and f-met-leu-phe. J Immunol 1990;145:2581-7.
31. De Simone C, Ferrari M, Ferrareli G, Rumi C, Pugnaloni L, Sorice F. The effects of substance P on human eosinophil receptors and functions. Ann N Y Acad Sci 1987;496:226-32.
32. Wiedermann F, Kähler C, Reinish N, Wiedermann C. Induction of normal human eosinophil migration in vitro by substance P. Acta Haematol 1993;89:213-5.
33. Chen X, Catravas JD. Neutrophil-mediated endothelial angiotensin-converting enzyme dysfunction: role of oxygen-derived free radicals. Am J Physiol 1993;265:L243-9.
34. Friedland J, Setton C, Silverstein E. Angiotensin converting enzyme: induction by steroids in rabbit alveolar macrophages in culture. Science 1977;197:64-5.
35. Katayama M, Nadel JA, Piedemonte G, McDonald DM. Peptidase inhibitors reverse steroid-induced suppression of neutrophil adhesion in rat tracheal blood vessels. Am J Physiol 1993;264:L316-22.
36. Piedimonte G, McDonald DM, Nadel JA. Neutral endopeptidase and kininase II mediate glucocorticoid inhibition of neurogenic inflammation in the rat trachea. J Clin Invest 1991;88:40-4.
37. Ichinose M, Barnes P. Bradykinin-induced airway microvascular leakage and bronchoconstriction are mediated via a bradykinin B2 receptor. Am Rev Respir Dis 1990;142:1104-7.
38. Corfield D, Webber S, Hanafi Z, Widdicombe J. The actions of bradykinin and lys-bradykinin on tracheal blood flow and smooth muscle in anesthetized sheep. Pulm Pharmacol 1991;4:85-90.
39. Baraniuk JN, Lundgren JD, Mizoguchi H, Peden D, Gawin A, Merida M, et al. Bradykinin and respiratory mucous membranes. Analysis of bradykinin binding site distribution and secretory responses in vitro and in vivo. Am Rev Respir Dis 1990;141:706-14.